PKA/AKAP/VR-1 Module: A Common Link of G_s-Mediated Signaling to Thermal Hyperalgesia

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Inflammatory mediators not only activate "pain-"sensing neurons, the nociceptors, to trigger acute pain sensations, more important, they increase nociceptor responsiveness to produce inflammatory hyperalgesia. For example, prostaglandins activate G_s-protein-coupled receptors and initiate cAMP- and protein kinase A (PKA)-mediated processes. We demonstrate for the first time at the cellular level that heat-activated ionic currents were potentiated after exposure to the cAMP activator forskolin in rat nociceptive neurons. The potentiation was prevented in the presence of the selective PKA inhibitor $PKI_{14–22}$, suggesting PKA-mediated phosphorylation of the heat transducer protein. PKA regulatory subunits were found in close vicinity to the plasma membrane in these neurons, and PKA catalytic subunits only translocated to the cell periphery when activated. The translocation and the current potentiation were abolished in the presence of an A-kinase anchoring protein (AKAP) inhibitor. Similar current changes after PKA activation were obtained from human embryonic kidney 293t cells transfected with the wild-type heat transducer protein vanilloid receptor 1 (VR-1). The forskolin-induced current potentiation was greatly reduced in cells transfected with VR-1 mutants carrying point mutations at the predicted PKA phosphorylation sites. The heat transducer VR-1 is therefore suggested as the molecular target of PKA phosphorylation, and potentiation of current responses to heat depends on phosphorylation at predicted PKA consensus sites. Thus, the PKA/AKAP/VR-1 module presents as the molecular correlate of G_s-mediated inflammatory hyperalgesia.

Key words: nociception; capsaicin; vanilloid receptor; sensitization; inflammation; sensory neuron

Apart from exciting nociceptors, inflammatory mediators increase nociceptor responsiveness, frequently by initiating G-protein-mediated processes, to cause hyperalgesia. In the $G_{q/11}$-mediated signaling cascade, a number of direct or indirect mechanisms have been identified that modulate the heat-transducing capsaicin-sensitive vanilloid receptor VR-1 (Cesare et al., 1999; Premkumar and Ahern, 2000; Chuang et al., 2001; Tominaga et al., 2001). In contrast, evidence for VR-1 as the molecular target affected by G_s signaling and the cAMP/protein kinase A (PKA) cascade is still controversial (Lee et al., 2000; De Petrocellis et al., 2001). Recent studies demonstrate that proinflammatory prostaglandin E_2 (PGE_2) induces sensitization of sensory neuron responses to heat (heat hyperalgesia) by activating G_s-coupled prostaglandin E (EP) receptor subtypes (EP3C and EP4) and subsequently the cAMP/PKA cascade (Kumazawa et al., 1996; Southall and Vasko, 2001). Accordingly, sensitization to heat also occurs in the presence of membrane-permeant cAMP analogs activating PKA (Kress et al., 1996). Moreover, mice carrying a null mutation for type Iβ PKA regulatory subunit (PKA-RIβ) no longer exhibit increased heat-induced pain behavior after PGE_2 administration, suggesting a crucial role of the cAMP/PKA second-messenger system in G_s-mediated hyperalgesia (Malmberg et al., 1997). First evidence suggesting a contribution of VR-1 in this pathway came from isolated sensory neurons in which capsaicin-activated ionic currents became facilitated in the presence of the adenylyl cyclase (AC) activator forskolin (FSK) (Lopshire and Nicol, 1998). In cellular models, PKA action crucially depends on a functional anchoring of the enzyme to its target via a specific group of A-kinase anchoring proteins (AKAP) (Dell'Acqua and Scott, 1997; Colledge and Scott, 1999). Such AKAPs have been found to target PKA to ion channels, e.g., glutamate receptors or voltage-dependent calcium channels in neurons (Rosenmund et al., 1994; Gray et al., 1998; Davare et al., 1999). A role for AKAPs in the targeting of PKA to the heat transducer VR-1 so far has not been reported.

In the present study, we investigated FSK effects on heat-activated ionic currents in sensory neurons, as well as in human embryonic kidney 293 (HEK293) cells transfected with wild-type VR-1. The importance of PKA consensus sites was determined with the help of VR-1 mutant channels. In addition, the expression of PKA subunits in sensory neurons and PKA coupling to heat transduction via AKAP was addressed.

MATERIALS AND METHODS

Culture of rat sensory neurons. Detailed dissociation procedures have been published previously (Zeilhofer et al., 1997; Haberberger et al., 2000). Briefly, lumbar dorsal root ganglia (DRG) were harvested from female inbred Wistar rats (100–160 gm) and transferred into DMEM

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Table 1. Gene-specific primer sequences

Gene	Forward primer	Reverse primer
PKA-C	5′-ACCTTGGGAACGGGTTCCTTCG-3′	5′-TACACCCAATGCCCACCAGTCC-3′
PKA-RI	5′-CAGCTACCGGAGAATCCTCATGGG-3′	5′-ATCTGAGCATGGGCCAAGGACG-3′
PKA-RII	5′-ACCTCAGACGGCTCCCTTTG-3′	5′-CGTCTCCAACCGCATAAGCAG-3′
dAKAP2	5′-AATGGCCTCCTCGCTGATCACC-3′	5′-AAGGCACGCAGCTCAAACTTGG-3′
AKAP-KL	5′-GTTTGAGCTGCGTGCCTTCCAC-3′	5′-GCGTGCAGCCGAGAAGTCAATC-3′
AKAP79	5′-AAAGAAAGCAGCCAAAGCAC-3′	5′-TGCCTGATCATTCAATGG-3′
AKAP220	5′-CGGCACAGCAGGCTTTGTTTCG-3′	5′-CTGTTGTGGTGCTGCTTGGTCG-3′

(Invitrogen, Karlsruhe, Germany) supplemented with 50 μg/ml gentamycin (Sigma, Deisenhofen, Germany). After removal of connective tissue, DRGs were treated with collagenase (0.28 U/ml in DMEM, 75 min; Roche Biochemicals, Mannheim, Germany) and trypsin (25,000 U/ml in DMEM, 12 min; Sigma). After dissociation and plating on glass coverslips coated with poly-L-lysine (200 μg/ml; Sigma), the cells were cultivated in serum-free TNB 100 medium (Biochrom, Berlin, Germany) supplemented with penicillin–streptomycin (each 200 U/ml; Invitrogen), L-glutamine (2 mM; Invitrogen), and nerve growth factor (mouse NGF 7S, 100 ng/ml; Alomone Labs, Tel Aviv, Israel) at 37°C in a humidified atmosphere containing 5% CO_2.

Reverse transcription-PCR. Total RNA was isolated from adult female rat DRGs and HEK293 cells using RNazol reagent (WAK-Chemie, Bad Soden, Germany) and reverse transcribed into cDNA using MuLV Reverse Transcriptase (PerkinElmer Biosystems, Weiterstadt, Germany) as described previously (Haberberger et al., 2000). PCR was performed in a 50 μl reaction volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 150 μM dNTP, 0.3 μM each gene-specific primer (Table 1), and 1.25 U of AmpliTaq Gold (PerkinElmer Biosystems) in the following amplification conditions: initial denaturation at 94°C for 5 min once, 94°C for 45 sec, 58°C for 30 sec, and 72°C for 45 sec for 35 cycles, followed by a 7 min extension at 72°C. The amplified fragments were cloned in TOPO vector (Invitrogen) and sequenced on the Applied Biosystems 373 DNA sequencer using *Taq* DyeDeoxy Terminator cycle sequencing kits (PerkinElmer Biosystems) to confirm the identity of the amplified products.

Indirect immunocytochemistry. Cells were fixed for 15 min with Zamboni's fixative (150 ml of saturated picric acid, 20 gm of paraformaldehyde, and 850 ml of phosphate buffer, pH 7.4) (Haberberger et al., 2000). Indirect immunofluorescence was performed for detecting vanilloid receptor VR-1 (1:1000) (Tominaga et al., 1998) and protein kinase A subunits using primary monoclonal IgG immune sera anti-RI, anti-RIIα, anti-RIIβ, anti-AKAP79, anti-AKAP149, and anti-AKAP220 (all 1:100; BD Transduction Labs, Hamburg, Germany) applied in the presence of 10% fetal bovine serum, 0.5% Triton X-100, 1% normal goat serum, and human Ig (Cohn's fraction II, 2 mg/ml; Sigma) in PBS for 24 hr at 4°C. Appropriate secondary antibodies coupled to Alexa488 (Molecular Probes, Leiden, The Netherlands) or Cy3 (Dianova, Hamburg, Germany) were applied in the presence of 1% normal goat serum and human Ig in PBS for 30 min at room temperature. After washing, the coverslips were mounted on glass slides with glycerol jelly (Merck, Darmstadt, Germany) and were analyzed with confocal laser scanning microscopy [Bio-Rad (Hercules, CA) MRC 1000 attached to a Nikon (Tokyo, Japan) Diaphot 300]. Alexa488 was excited with the 488 nm line of a krypton–argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT). Single confocal optical sections were obtain with a 60× oil immersion objective (numerical aperture 1.4). The length/profile function of COMOS software (Bio-Rad) was used to quantify peripheral translocation of PKA immunostaining. The total average fluorescence intensity over the cell diameter (F) was calculated and set to 1. To quantify the redistribution of PKA catalytic subunit (PKA-C), average fluorescence intensities were calculated for 10% segments of the total intensity profile length (ΔF), normalized to F ($\Delta F/F$), and compared between peripheral and central regions of the cell.

Site-directed mutagenesis and transfection. VR-1 cDNA and anti-VR-1 antibody were a generous gift from David Julius (University of California, San Francisco, CA). Mutagenesis of VR-1 was performed with VR-1 cDNA subcloned into pcDNA3 by means of the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) using a mutagenesis primer and a restriction primer. In the restriction primer, the wild-type *Hind*III site has been changed to *Ssp*I. *In vitro* synthesis was performed for 2 hr, with one addition of dNTPs and T4-DNA polymerase during the reaction. Potential mutants were identified by restriction enzyme digestion as *Hind*III-resistant plasmids and confirmed by DNA sequencing with appropriate primers near the mutated region.

HEK293t cells were transfected with VR-1-pcDNA3 (2 μg) and reporter plasmid CD8-pih3m (1 μg) by the calcium phosphate precipitation method (Cannon and Strittmatter, 1993). After incubation for 12–15 hr, cells were replated in 35 mm culture dishes. Transfected cells were used for experiments within 3 d. Transfection-positive cells were identified by immunobeads (CD-8 Dynabeads; Dynal, Oslo, Norway).

Electrophysiology. Recordings from neurons or HEK293t cells were performed in external solution (ECS) containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES buffer, pH 7.3. Whole-cell voltage-clamp current measurements were performed at −80 mV holding potential using an Axopatch amplifier and pClamp 6.0 (Axon Instruments, Foster City, CA). Borosilicate electrodes (Science Products, Hofheim, Germany) contained 145 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 2 mM Na-ATP, 0.2 mM Li-GTP, 10 μM fura-2 pentapotassium salt, and 10 mM HEPES buffer, adjusted to pH 7.3 with KOH (final resistance, 2–3 MΩ). Forskolin, PKI_{14-22} (both from Calbiochem, Nottingham, UK), InCELLect, and control peptide (both from Promega, Mannheim, Germany) were dissolved in internal solution (ICS) to the final concentration. For drug application and heat stimulation, a fast seven-channel system with common outlet was used as described previously (Dittert et al., 1998; Guenther et al., 1999; Kress and Guenther, 1999).

Data analysis. For detailed statistical analysis, the CSS software package was used (StatSoft, Tulsa, OK). All summarizing results are given as means ± SEM. For intra-individual data comparisons, the Wilcoxon matched pairs test was calculated, if not stated otherwise, and differences were considered significant at $p < 0.05$.

RESULTS

Activation of the cAMP/PKA cascade potentiates I_{heat} in DRG neurons

To determine whether the activation of PKA was relevant for heat sensitization, electrophysiological recordings of single capsaicin-sensitive neurons from DRG in culture were performed. At −80 mV holding potential, heat-activated ionic currents (I_{heat}) were elicited from a threshold temperature of 43.9 ± 1.4°C that neither sensitized nor desensitized during repetitive stimulation at 1 min intervals with the stimulus strength used. I_{heat} significantly increased from 480 ± 154 to 779 ± 169 pA immediately after FSK application (10 μM) (Fig. 1*a*), and activation threshold of I_{heat} significantly dropped from 43.9 to 41.6 ± 1.0°C ($n = 7$; $p < 0.05$). On average, these plastic changes fully recovered within 2 min. The inactive dideoxy-FSK control was ineffective. To prove that the full cAMP/PKA pathway contributed to the sensitization of I_{heat}, the selective PKA inhibitor protein PKI_{14-22} was added to the pipette solution in a number of experiments. Under this condition, the FSK-induced potentiation of I_{heat} was totally suppressed (10 μM) (Fig. 1*c*). This supports a role of the cAMP/PKA cascade in the heat sensitization process of nociceptors.

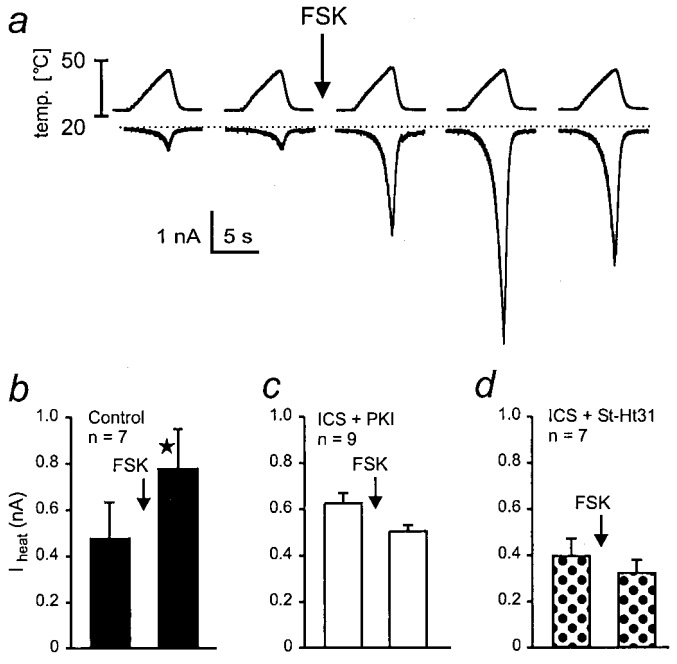

Figure 1. FSK-induced potentiation of native heat-activated ionic currents (I_{heat}). *a*, Example of a DRG neuron that was repetitively stimulated with noxious heat (linear temperature rise from room temperature to 46°C, 5 sec duration, 1 min intervals) before and after FSK (10^{-5} M). *b–d*, Mean ± SEM responses before and immediately after FSK, and FSK with the selective PKA inhibitor peptide PKI_{14-22} or the InCELLect AKAP St-Ht31 inhibitor peptide in the patch pipette. Peak stimulation temperatures were similar in the different samples (47.0 ± 1.5°C in controls; 46.7 ± 0.6°C for PKI; 47.2 ± 0.9°C for StHt31). ⋆$p < 0.05$ indicates significant differences.

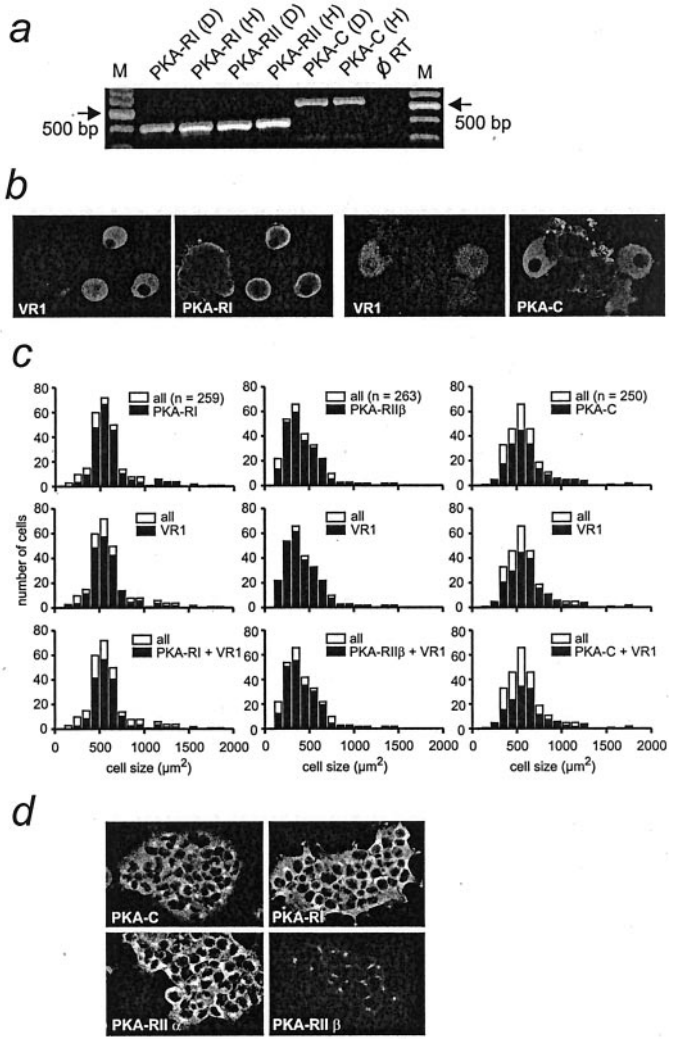

Figure 2. Expression of VR-1, PKA subunits, and AKAPs in DRG-neurons. *a*, RT-PCR products for PKA-RI, PKA-RII, and PKA-C of total mRNA isolated from DRG (*D*) or HEK293t cells (*H*) and negative control without reverse transcriptase (*ØRT*). *M*, Marker. *b*, Indirect immunocytochemistry for VR-1 and PKA subunit, respectively. Cells were fixed and stained with antibodies against VR-1 and PKA-RI, RII, and PKA-C, respectively. *c*, Size distribution of neurons in culture double labeled for PKA-RI and VR-1 and PKA-C and VR-1, respectively. The cell size was determined off-line with the noncommercial image processing software IPB by Marc Nischik (Institute of Physiology, Erlangen). *d*, Immunostaining of HEK293t cells for different PKA subunits.

mRNA expression and immunocytochemical localization of PKA subunits in DRG neurons

Reverse transcription (RT)-PCR revealed mRNA expression for regulatory PKA subunits RI and RII, as well as for catalytic subunit in sensory ganglia from adult rat (Fig. 2*a*). To obtain information on the subcellular localization of the enzyme, we performed indirect immunofluorescence in sensory neuron cultures. The immunostaining revealed the presence of PKA subunits in neurons and a high degree of coexpression with VR-1: VR-1-positive neurons showed an almost full overlap with immunoreactivity for PKA regulatory subunit RI and RII, as well as catalytic subunit (Fig. 2*c*). Thus, RI, as well as RII, subunits may be part of the signaling complex in sensory neurons as reported previously (Malmberg et al., 1997).

Confocal laser scanning microscopy showed that PKA subunits exhibited a differential distribution in neurons with the regulatory RI subunit in close vicinity to the plasma membrane. In contrast, catalytic subunit staining was evenly distributed throughout the cytoplasm in nonstimulated cells (Fig. 2*b*).

Forskolin-induced translocation of catalytic PKA subunit in DRG neurons and effects of AKAP St-Ht31 inhibitor peptide

Stimulation of AC/PKA with FSK translocated catalytic subunit to the periphery of the cell in the majority of small-diameter neurons (28 of 30 from three different dishes) (Fig. 3*a*,*b*). Such translocation was significantly less frequent with the inactive analog dideoxy-FSK (2 of 30) (Fig. 3*e*). To determine the time course of the translocation, cells were allowed to recover in normal ECS. Confocal line profile analysis yielded that the translocation was transient and recovered completely within 60 sec in all cells investigated (Fig. 3*c*). Because PKA anchoring to its target requires specific AKAPs, FSK stimulation was performed in cultures preincubated with the InCELLect AKAP St-Ht31 inhibitor peptide, which disrupts the spatial coupling of PKA via AKAP (Vijayaraghavan et al., 1997). Preincubation of sensory neurons with the InCELLect AKAP St-Ht31 inhibitor peptide but not the inactive control peptide St-Ht31P prevented the translocation of PKA-C subunit (Fig. 3*d*).

This finding was corroborated by correlative electrophysiological recordings from sensory neurons: the FSK-induced potentiation of I_{heat} was also significantly reduced when the InCELLect AKAP St-Ht31 inhibitor peptide was added to the ICS in the

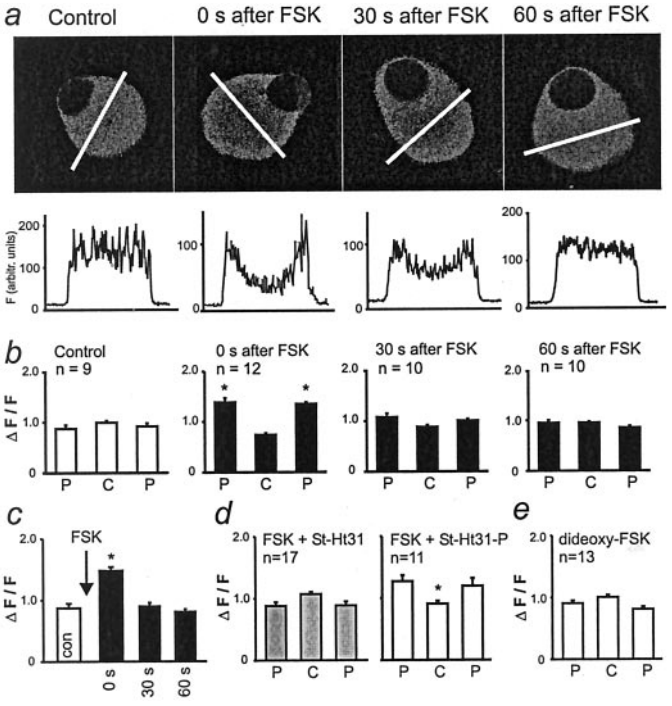

Figure 3. Translocation of PKA-C after FSK stimulation (35 sec, 10^{-5} M) in DRG neurons. *a*, Examples of neurons stained without or after FSK stimulation. Confocal images indicate the position of the line scan profile used for calculations in *b*. *b*, Confocal line scan profile taken with the COMOS software. *c*, *Columns* show the average fluorescence magnitude in the peripheral and central regions of the cell diameter divided by the total average fluorescence of the cell. *con*, Control. *d*, *e*, Time course of the PKA-C translocation after FSK, depicted as the difference between peripheral (*P*) and central (*C*) fluorescence and effects of the selective inhibitors PKI_{14-22}, InCELLect AKAP inhibitor peptide plus inactive control peptide, and the inactive analog 1,9-dideoxy-FSK.

patch pipette to disrupt PKA coupling to heat-activated ion channels (Fig. 1*d*). This suggests that, in nociceptors, PKA-mediated potentiation of heat-activated ionic channels depends on spatial coupling of the enzyme to its target via AKAPs. We found a number of PCR products of AKAPs in rat dorsal root ganglia (Fig. 4*a*). RT-PCR revealed expression of the vesicular AKAP220, as well as of AKAP79 (which is located in the plasma membrane and preferentially binds RII PKA subunit). In addition, mRNA for the dual dAKAP2 (binding RI and RII subunits with comparable affinity) was detected. Indirect immunocytochemistry revealed expression of AKAP220 and AKAP79 immunoreactivity in practically all neurons. In ~80% of AKAP220-expressing cells and ~90% of AKAP79-expressing cells, VR-1 immunoreactivity was colocalized (Fig. 4*b*). For dAKAP2 no antibodies were available. Although we cannot identify which AKAP contributes to PKA-mediated heat sensitization with the tools that are presently available, it may be concluded that sensory neurons possess the complete machinery for this signaling pathway.

PKA-mediated phosphorylation of VR-1 potentiates I_{heat} in HEK293t cells

Because the multimodal signal transducer VR-1 has been suggested to be one major constituent of the heat transduction process in nociceptors, whole-cell voltage-clamp recordings were performed in HEK293t cells transiently transfected with VR-1, and FSK effects were investigated. HEK293t cells constituently

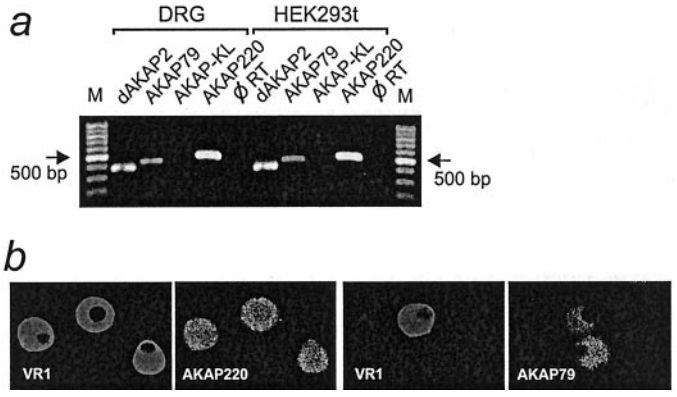

Figure 4. Sensory neurons and HEK293t cells express AKAPs. *a*, Expression of mRNA for dAKAP2, AKAP79, and AKAP220 but not AKAP-KL in DRGs and HEK293t cells and negative control without RT (ø*RT*). The expected size of the fragments is between 400 and 510 bp. *M*, Marker. *b*, Immunostaining of DRG neurons in culture reveals expression of VR-1 together with AKAP79 or AKAP220.

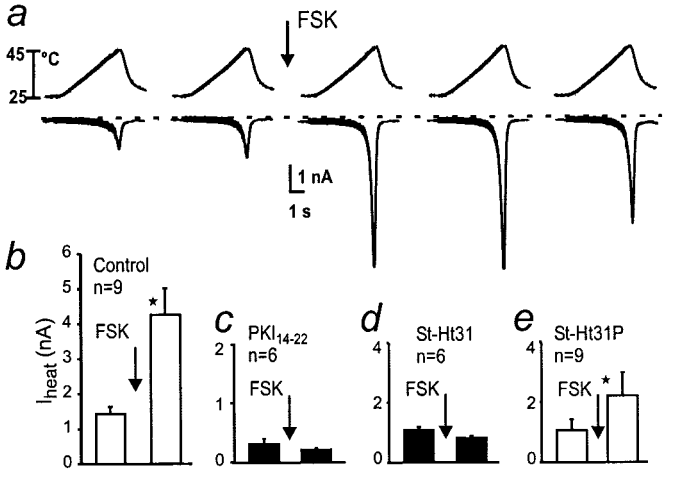

Figure 5. FSK-induced potentiation of heat-activated currents in VR-1-transfected HEK293t cells. *a*, Whole-cell current responses evoked by repeated noxious thermal stimuli before and after a pretreatment period with FSK (10^{-5} M) for 1 min. The interval between two thermal stimuli was 1 min. Heat response (mean ± SEM) before and after FSK pretreatment (*b*) and in the presence of the selective PKA inhibitor PKI_{14-22} (*c*), or the InCELLect AKAP St-Ht31 inhibitor peptide in the ICS (*d*,*e*).

express regulatory, as well as catalytic, PKA subunits, as demonstrated by RT-PCR in Figure 1*a*. Similar to sensory neurons, FSK-induced PKA activation resulted in a considerable increase of the current response to heat (1.4 ± 0.2 to 4.3 ± 0.7 nA; $p < 0.01$) (Fig. 5*a*,*b*), which was prevented by intracellular application of the selective PKA inhibitor peptide PKI_{14-22} via the patch pipette (Fig. 5*c*). Intracellular equilibration with the InCELLect AKAP St-Ht31 inhibitor peptide (but not the inactive control peptide) also inhibited current potentiation by FSK completely (Fig. 5*d*,*e*). We also found that HEK293t cells expressed the same AKAPs that we found previously in sensory ganglia (Fig. 4*a*). VR-1 thus seems to resemble a potential target of PKA phosphorylation and may therefore account for the changes in I_{heat} induced by FSK in sensory neurons.

To determine whether the potentiation was attributable to direct phosphorylation of VR-1, we generated mutants of the three predicted PKA phosphorylation sites, T144, T370, and

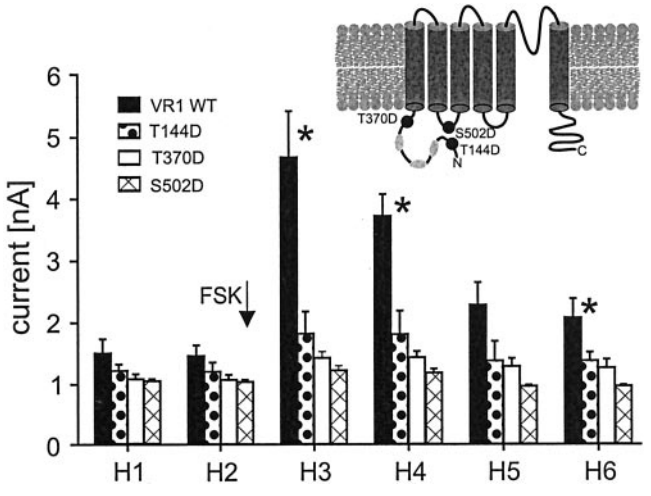

Figure 6. Mean heat responses of wild-type and mutant VR-1 expressed in HEK293t cells before and after FSK pretreatment ($n = 7–9$; $p < 0.05$). * indicates statistically significant difference of current potentiation after FSK in mutants compared with wild-type. Heat stimuli H1 through H6 were applied at 1 min intervals.

S502, in which threonine or serine at these sites were exchanged by aspartate. When transfected into HEK293t cells, in all three mutations, heat-activated inward currents were preserved. FSK-induced potentiation, however, was considerably impaired. Mutations T144D and T370D were less affected, whereas in mutation S502D, the lack of FSK-induced potentiation of the heat response was most pronounced (Fig. 6). Also, in mutants in which the sites were exchanged by alanine, FSK did not induce a sensitization of heat-activated currents (data not shown).

DISCUSSION

For the first time, we present evidence that activation of the cAMP/PKA cascade with FSK potentiates heat-activated ionic currents in DRG neurons. Similar results were obtained in HEK293t cells expressing wild-type VR-1, whereas the FSK-induced current potentiation was considerably reduced in point mutations of the predicted PKA phosphorylation sites. Our results suggest potentiation of heat-activated VR-1 currents by PKA phosphorylation as the mechanism of PKA-mediated heat sensitization. We also demonstrate the functional expression of regulatory and catalytic PKA subunits, as well as of a number of AKAPs, in sensory neurons. Exposure of neurons to FSK induced a transient and reversible translocation of PKA catalytic subunit to the cell periphery and a PKA-mediated potentiation of heat-activated ionic currents with similar time course. Both PKA translocation and potentiation of I_{heat} were blocked by an AKAP inhibitor peptide.

A number of inflammatory mediators that cause inflammatory pain and hyperalgesia exert their effect via G_s-coupled membrane receptors at the cellular level. Among these mediators, prostaglandins are known to use the cAMP/PKA signaling cascade to sensitize nociceptors to heat (Mizumura et al., 1993; Hingtgen et al., 1995; Kress et al., 1996; Southall and Vasko, 2001). VR-1 was identified as one transducer of heat nociception in peripheral nociceptive nerve terminals (Caterina et al., 1997, 2000; Davis et al., 2000). Today, it is generally accepted that VR-1 can be sensitized so that current activation even occurs at ambient temperature by membrane receptor activation using $G_{q/11}$ protein, followed by phospholipase C and protein kinase C (PKC) activation (Premkumar and Ahern, 2000; Chuang et al., 2001). In addition, VR-1 was suggested as a potential target of PKA phosphorylation for two reasons: first, capsaicin-activated ionic currents were potentiated after FSK stimulation (Lopshire and Nicol, 1998), and, second, in mice carrying a null mutation for VR-1, thermal hyperalgesia after inflammation was greatly reduced (Caterina et al., 2000; Davis et al., 2000). More hints toward the relevance of PKA phosphorylation of VR-1 also came recently from a biochemical study (De Petrocellis et al., 2001). In the present study, we, for the first time at the cellular level, demonstrate an FSK-induced potentiation of I_{heat} in sensory neurons that was abolished by the selective PKA inhibitor $PKI_{14–22}$. To further address the target of PKA phosphorylation, we expressed VR-1 in HEK293t cells and examined I_{heat} before and after exposure to FSK. HEK293t cells expressing wild-type VR-1 exhibited a potentiation of heat-activated currents after exposure to FSK that was similar to the one obtained in sensory neurons. It was also blocked by $PKI_{14–22}$ or by disruption of AKAP anchoring (see below). The FSK effect was drastically reduced in mutant VR-1 in which PKA consensus sites were mutated. This suggests that FSK-induced current potentiation is mediated by a direct phosphorylation of the VR-1 channel protein at the predicted consensus sites. Because the S502D mutant showed the most prominent reduction in current potentiation, phosphorylation at this site seemed to be most important to determine the channel properties. It is located in the linker between the second and the third transmembrane domain. Our knowledge about the structure function relationship is still very limited. However, the negative charge that is introduced by phosphorylation of the site may affect other charges inside the channel pore to increase channel conductance or open probability, as suggested for capsaicin-induced currents (Lopshire and Nicol, 1998).

In contrast to PKC, which is considered a classical translocation enzyme involved in heat sensitization (Cesare et al., 1999), translocation of PKA catalytic subunits to the cell periphery is a new finding. If at all, translocation of regulatory subunits from the cell membrane into the cytosol or of catalytic subunits to the nucleus has been reported previously (Hagiwara et al., 1993; Dohrman et al., 1996; Feliciello et al., 2000). The present study for the first time reveals a translocation of catalytic subunit toward the cell periphery during exposure to FSK, which depends on functional AKAP and which may be specific to sensory neurons. Since the first PKA anchor protein microtubule-associated protein-2 was detected (Theurkauf and Vallee, 1982), numerous AKAPs have been identified from diverse species and tissue. A number of AKAPs have been cloned that bind regulatory PKA subunits to target PKA in proximity to transmembrane proteins that become phosphorylated only when this anchoring is maintained (Dell'Acqua and Scott, 1997; Colledge and Scott, 1999). Most of the AKAPs identified so far preferentially bind RII subunit, and some of the AKAPs (e.g., Yotiao or AKAP15/18) directly target PKA to ion channels (Colledge and Scott, 1999). Few AKAPs exhibit dual specificity binding to RI, as well as RII, subunits, e.g., AKAP-KL or dAKAP1 and dAKAP2 (Huang et al., 1997a,b; Colledge and Scott, 1999). Our results provide evidence that members of this dual AKAP subfamily may be the appropriate candidates for coupling PKA to VR-1 for phosphorylation of the channel and potentiation of the heat responses. More interesting, some of the dual AKAPs also couple PKC and calcineurin to ion channels (Colledge and Scott, 1999). One may speculate that they form a signaling complex in the cell membrane that targets kinases and phosphatases to VR-1 and yield a complex modulator

array at the internal channel site (Docherty et al., 1996; Cesare et al., 1999). A lack of the appropriate PKA/AKAP machinery might be responsible for the previously reported lack of PKA effects in *Xenopus* oocytes or *Aplysia* R2 neurons (Ali et al., 1998; Lee et al., 2000).

In summary, we demonstrate that FSK stimulation of nociceptive neurons induces a transient and reversible translocation of PKA catalytic subunit to the cell periphery and a potentiation of heat-activated ionic currents. Both effects depend on functional anchoring of PKA. We show that both PKA and AKAPs are coexpressed with VR-1. Similar results were obtained in HEK293t cells expressing wild-type VR-1. FSK-induced current potentiation was considerably reduced in point mutations of the predicted PKA phosphorylation sites, suggesting potentiation of VR-1 currents by PKA phosphorylation. We conclude that the PKA/AKAP/VR-1 module represents the molecular target of G_s-coupled receptors to cause thermal hyperalgesia.

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